

miR-23a: A Key Metabolic Regulator in Hepatocellular Carcinoma

Undergraduate Honors Thesis

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Abstract

Considerable efforts have been made in elucidation of the mechanisms that drive cancer metabolism towards high levels of anaerobic glycolysis.¹ We have demonstrated that the expression and activity of key enzymes within the gluconeogenesis pathway are drastically reduced in human and mouse hepatocellular carcinoma (HCC), which is at least partially due to a significant upregulation of microRNA-23a (miR-23a).² We have further shown that the messenger RNAs (mRNA) coding for two vital gluconeogenic proteins, PGC-1 α (the major transcription coactivator for key gluconeogenic enzymes) and glucose-6-phosphatase (G6PC), are direct targets of miR-23a.² This was the first report that demonstrated the role of a specific miRNA in regulation of gluconeogenesis in HCC.² Although these findings have established a key role for miR-23a in gluconeogenesis, its potential for the regulation of other metabolic pathways requires further investigation. To explore this possibility, we utilized Phenotype MicroArrayTM technology to determine the effects of miR-23a expression on the cellular metabolism of nearly one hundred different metabolites. This analysis revealed a significant role of miR-23a in the inhibition of citrate metabolism. mRNA coding for Aconitase (ACO1), a key enzyme involved in the citric acid cycle, has a potential binding site for miR-23a in its 3' untranslated region (UTR). We confirmed the attenuation of ACO1 by miR-23a through real-time RT-PCR and 3' UTR luciferase reporter assays. These results indicate that significant up-regulation of miR-23a may play an essential role in the shift of cancer cell metabolism towards high levels of anaerobic glycolysis and nucleotide synthesis through the accumulation of glucose-6-phosphate.

Background

In 1956, Otto Warburg demonstrated that proliferating cancer cells prefer to consume glucose through lactic acid formation rather than oxidative phosphorylation, differing from glucose consumption in noncancerous tissues.³ This first observation of altered metabolism in cancerous cells was not pursued further for decades. Until recently, cancer research has focused on the study of genetic mutation, rather than deregulated metabolism.⁴

There are several reasons for a lack of interest in Warburg's discovery over the past several decades. First, the conversion of glucose to lactate is highly inefficient.¹ This metabolic pathway only produces two molecules of adenosine triphosphate (ATP) per molecule glucose; comparatively, when one molecule of glucose is consumed through oxidative phosphorylation, 36 molecules of ATP can be recovered.⁵ Second, it was unknown how such an alteration in metabolism could contribute to the cancer phenotype. Third, the mechanism to mediate this drastic shift in metabolism has been poorly understood.¹ Lastly, the relationship between the onset of cancer, through the mutual activation of oncogenes and inactivation of tumor suppressor genes, and metabolism deregulation was not recognized.¹

Over the past decade, there has been an increase in efforts to understand the functional significance of the altered glucose metabolism pathway in cancerous cells. However, the possibility of an altered gluconeogenesis pathway, the metabolic pathway responsible for glucose production, has only been explored recently, largely due to work in our laboratory.² Gluconeogenesis is a vital pathway in all organisms; it is the exclusive supplier of the body's glucose while fasting.⁵

A viable mouse model for hepatocellular carcinoma is available which can induce liver tumors by feeding the mice a choline-deficient amino-acid defined (CDAA) diet. The CDAA diet induced HCC mouse model mimics all of the hallmark stages of tumorigenesis including steatosis, fibrosis and tumor formation in the absence of chemical carcinogens. This diet induced mouse model was utilized in our laboratory to determine the potential modifications to the gluconeogenesis pathway.² HCC is the ideal system to determine alterations in gluconeogenesis: the liver is the primary site of gluconeogenesis in the body, and consequentially, contains all of the enzymes essential for gluconeogenesis.^{2, 5} The rate of gluconeogenesis in the liver is controlled by three crucial enzymes: glucose-6-phosphatase (G6PC), phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-biphosphatase (FBP1).⁶ Past experiments demonstrated that expression and activity

of all three of these key enzymes is significantly suppressed in HCC tissue.⁵ This is of vital significance because more than 80% of body's G6PC is present in the liver and all three of the reactions associated with these enzymes are irreversible.^{2, 5} Thus, glucose production in HCC is severely inhibited. In addition to these findings, expression of an important transcription coactivator, Peroxisome Proliferator-Activated Receptor- γ Coactivator-1 α (PGC-1 α), was also significantly reduced in cancerous tissues.² PGC-1 α interacts with transcription factors to aid in transactivation of G6PC and PEPCK.^{6, 7} Since PGC-1 α indirectly promotes the gluconeogenesis pathway, reduced levels of this protein further suppress glucose production in HCC.

Further research showed that mice fed a CDAA diet displayed increasing levels of mircoRNA 23a (miR-23a) with development of liver tumors.² MircoRNAs are small, noncoding ribonucleic acids consisting of 19-25 nucleotides.⁸ MicroRNA (miR) acts as a post-transcriptional regulator of genes through degradation of its target mRNA or inhibiting the translation of target mRNA.⁸ Further experimentation indicated that mRNA coding for PCG-1 α and G6PC are direct targets of miR-23a. This was the first report that demonstrated the role of a specific miR in regulation of gluconeogenesis in HCC. These findings from our lab have shown that miR-23a plays a key role in HCC gluconeogenesis; however, its potential for regulation on other metabolic pathways requires further investigation.²

Relevance

Global statistics published in 2011 report that hepatocellular carcinoma (HCC) is the fifth most common neoplasm in men and the seventh most common in women.⁹ However, due to late diagnosis of this cancer and lack of effective drugs for treatment, HCC is the 2nd most frequent cause of death from cancer in men.⁹ In the majority of cases (75-80%), HCC development is attributed to persistent viral infection of hepatitis B and C.¹⁰ With early diagnosis, the median survival rate of patients with HCC is beyond 5 years; however, the effect of treatment is very limited by both tumor size and location.¹¹ Liver transplantation proves to be a highly effective treatment for HCC, offering a 4-year survival rate of 75% in select patients. The possibility of a transplant is also diminished in patients with excess liver damage.¹² However, the shortage of liver donors makes this treatment inaccessible for most patients.¹¹ In order to improve the prognosis for patients with HCC, new treatments must be developed.

It is known that the by-products of metabolism, specifically, reactive oxidative species, can promote DNA mutations leading to tumorigenesis.¹³ Conversely, carcinogenic mutations can lead to changes in cellular metabolism,¹³ which facilitate growth and proliferation of cancer cells.^{4, 14} These alterations in metabolism create a therapeutic window for targeting cancer cells by exploiting the differences between normal and oncogenic metabolic enzymes.¹³ However, the complex regulation of cancer genes on metabolism has yet to be fully understood. If these multifaceted regulations can be better defined, novel anticancer therapeutics could be developed to target specific metabolic facilitators.¹³

Hypothesis and Objectives

Previous studies have shown that miR-23a plays a significant role in the regulation of HCC gluconeogenesis.² As an extension of this work, it is hypothesized that miR-23a also has strong effect on other key metabolic pathways. This research aims to enhance our current understanding of the role miR-23a plays in HCC metabolism.

- Specific Aim 1:* To utilize Phenotype MicroArrayTM technology to determine which metabolic pathways are strongly affected by miR-23a expression
- Specific Aim 2:* To elucidate the regulatory mechanisms of miR-23a mediated pathways by identifying key enzymes within the pathways that are either directly or indirectly regulated by miR-23a
- Specific Aim 3:* To confirm the phenotype microarray results using NMR based stable-isotope resolved metabolomics

Phenotype MicroArray

Overview - The Phenotype Microarray™ consists of multiple 96-well plates seeded with various carbon and nitrogen sources. Cells can be seeded into the phenotype microarray plates in glucose-free media. In the absence of any exogenous glucose, the cells have to rely on metabolizing the provided metabolite in each well, which is the only available energy source. A colorimetric dye is then added to each well. The dye is reduced by the NADH produced by the metabolic reaction resulting in changes of the absorbance of the media at 590 nm. The change in absorbance over time can be measured using a kinetic spectrometer. The rate of dye reduction is then correlated to rate of metabolism. The purpose of using the Phenotype Microarray™ was to determine which metabolic pathways were strongly affected by miR-23a. However, it was vital to optimize the Phenotype Microarray procedure prior to performing these experiments.

Optimization - Prior to performing a Phenotype Microarray™ (PM), it is important to optimize the number of cells seeded into each well and the length of incubation time prior to dye addition.¹⁵ After seeding cells into PM plates, the cells must be left to incubate for ~4-8 hours prior to dye addition. The incubation period should be long enough for the cells to metabolize all remaining intracellular energy sources, but not long enough to metabolize all the provided energy within the well. If the incubation period is too short, cells may still contain intracellular energy stores that were not obtained from the substrate provided within the well. The metabolism of these energy stores will cause unwanted dye reduction. Conversely, if the incubation period is too long, cells may have metabolized all of the provided energy within some wells prior to dye addition. This can lead to an unwanted decrease of dye reduction within these wells. The optimal incubation time will result in the highest ratio between the rate of dye formation in wells with high nutrition and wells with low nutrition. In order to simulate a well that provides low viable nutrition, cells were seeded with IF-M1 media (BioLog, Hayward Ca) with 5% dialyzed fetal bovine serum and 0.3 mM Glutamine.¹⁵ The IF-M1 media is comparable to RPMI media without glucose and phenol red. To simulate a well that provides high nutrition, cells were seeded with a standard media consisting of DMEM without phenol red, 10% fetal bovine serum, nonessential amino acids and 2.0 mM Glutamine. Figure 1 shows the net dye reduction in wells with low nutrition after various incubation periods. The ratios of rate of dye reduction in wells with high nutrition to wells with low nutrition can be seen in Table 1.

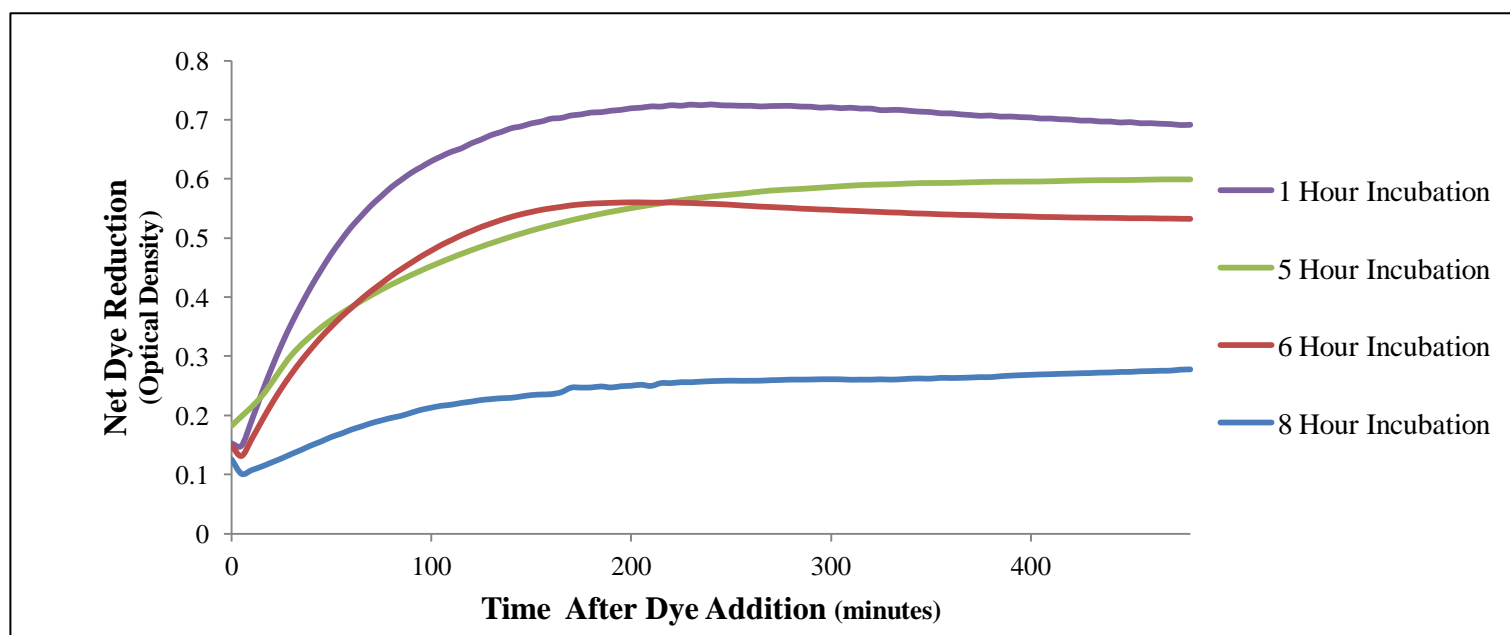


Figure 1: Net dye reduction in HepG2 Cells with low-nutrition medium at various incubation times – 20,000 cells/well

It can be seen from Table 1 that the maximum ratio results from an incubation time of 8 hours. This ratio will also increase by increasing the number of cells/well. However, seeding too many cells into each well can lead to non-linear dye reduction. It can be seen in Figure 2 that increasing the number of cells per well above 20,000 results in dye reduction out of the linear range. It is important to stay within the linear range of dye reduction so that the various rates of metabolism within the PM plates can be directly compared to each other. From this data, we determined that the optimal condition to run the PM with HepG2 cells is to seed 20,000 cells/well and incubate 8 hours prior to dye addition. A complete protocol for this optimization can be found attached in Appendix A.

Incubation Time	High : Low Nutrition Ratio
1	4.59
5	6.15
6	7.16
8	15.02

Table 1: Ratios of maximum rates of dye reduction in wells with high nutrition medium to those with low nutrition medium – HepG2 Cells, 20,000 cells/well

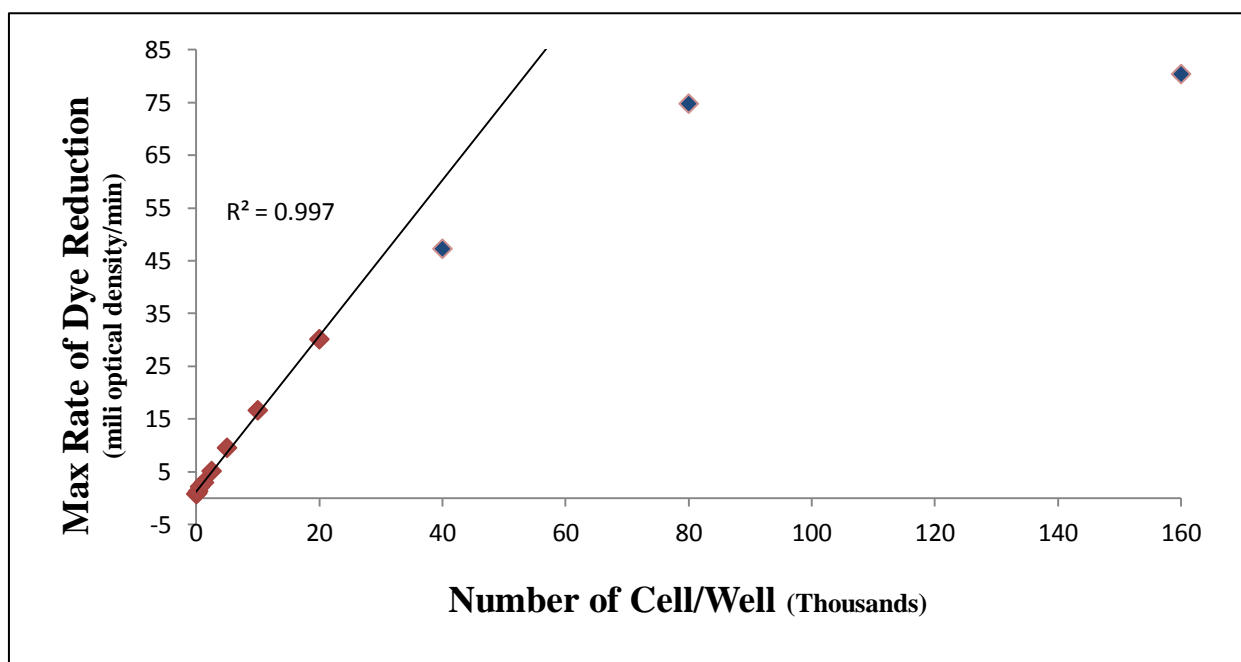


Figure 2: Rate of Dye Reduction from HepG2 in high nutrition medium - 5hr Incubation time

Phenotype MicroArrayTM Results - After the HepG2 cell line was optimized, the PM was used to determine which metabolic pathways were strongly affected by miR-23a. HepG2 cells transiently transfected with miR-23a or a negative control RNA were seeded into a PM plate with various carbon energy sources (PM-M1). The dye reduction was then measured with a dynamic spectrometer over two hours. The maximum rate of dye reduction was calculated for every well. The data collected from the miR-23a transfected cells was then compared to the control cells. This analysis revealed miR-23a significantly inhibited the ability of HepG2 cells to metabolize citrate, as seen in Figure 3.

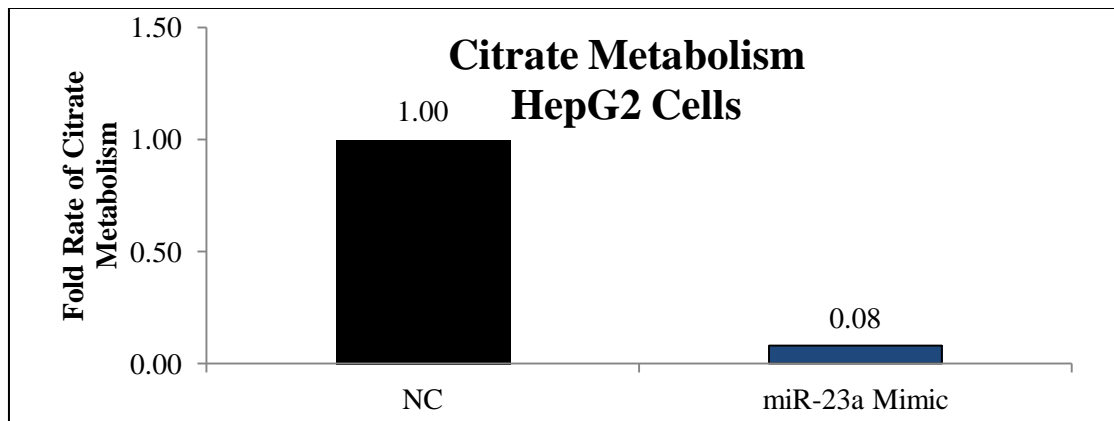


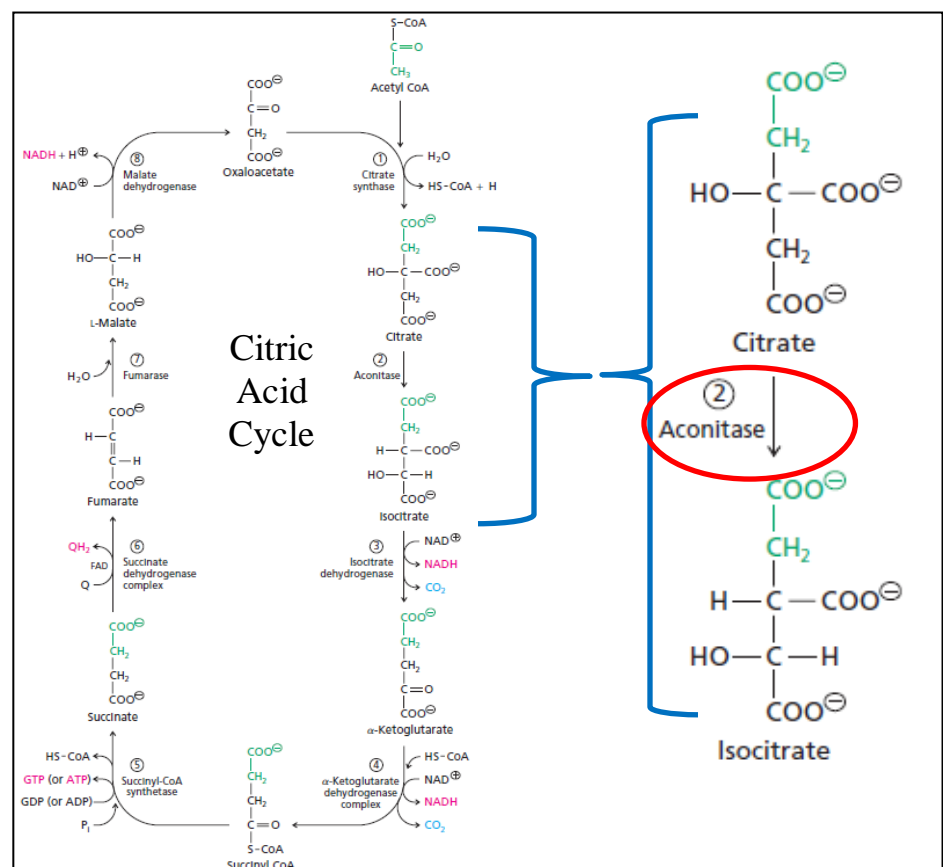
Figure 3: HepG2 cells transiently transfected with miR-23a mimic or a negative control (50 nM – 48 hours) were metabolically profiled using Phenotype MicroArrays™. This analysis revealed that citrate catabolism is suppressed from miR-23a overexpression

HepG2 was the only cell line utilized in the phenotype microarray due to its fast rate of metabolism. Optimization was attempted on other HCC cell lines (Huh7, Hep3B and SNU182); however, data analysis revealed that these cell lines were not capable of producing measurable rates of dye reduction.

miR-23a Targets Aconitase 1

Citrate Metabolism - Citrate is a metabolic intermediate within the citric acid cycle. An overview of the citric acid cycle and citrate metabolism can be seen in Figure 4. Due to the substantial inhibition of citrate metabolism observed in the phenotype microarray, it was hypothesized that miR-23a inhibits an enzyme involved in citrate metabolism. Aconitase 1 (ACO1) is a cytosolic enzyme responsible for the isomerization of citrate to isocitrate. Based on the microRNA target prediction algorithm used by TargetScan.org, ACO1 has a predicted miR-23a binding site on its 3' UTR. Due to this prediction, numerous experiments were performed to confirm that miR-23a is capable of directly suppressing ACO1 expression.

Figure 4: Citrate is a metabolic intermediate in the citric acid cycle. The enzyme aconitase is responsible for the isomerization of citrate to isocitrate. This figure is adapted from Moran and Laurence⁴



Real-Time PCR Results - RT-PCR was used to determine if miR-23a was capable of suppressing ACO1 at the transcriptional level. HepG2 and Hep3B cells were transiently transfected with miR-23a or a negative control RNA. RNA was harvested after 48 hours of transfection. Complementary DNA (cDNA) produced from the RNA templates was then used to analyze the expression of ACO1 compared to that of the housekeeping genes GAPDH and β -Actin. The normalized ACO1 expression of the miR-23a transfected cells was then compared to that of the control cells. As seen in Figure 5, ACO1 expression was significantly reduced in cells transfected with miR-23a.

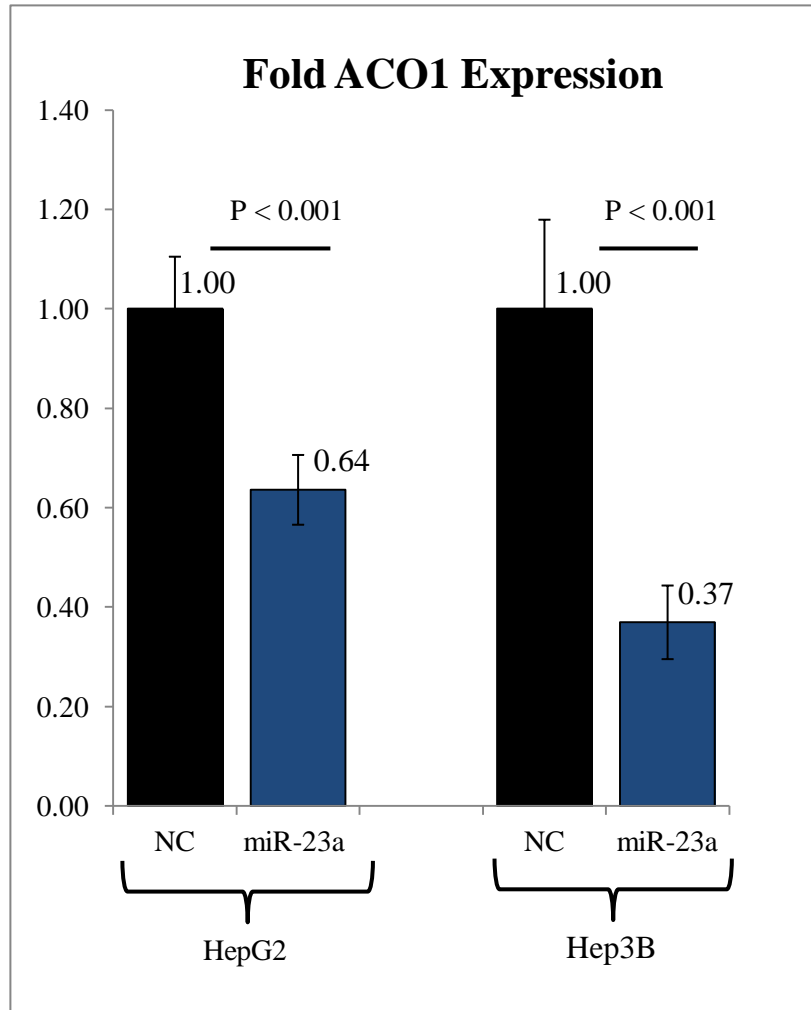


Figure 5: RT- PCR results. The expression of mRNA coding for ACO1 was quantified by Real time RT-PCR. The ACO1 mRNA level in HepG2 cells was significantly lower in cells transfected with miR-23a mimic (50 nM – 48 hours) compared to the corresponding negative control. This same trend was also observed in Hep3B cells.

3'UTR Luciferase Reporter Assay - Although the RT-PCR data suggests that miR-23a inhibits ACO1 expression, it does not prove that miR-23a directly targets the 3'UTR of ACO1. In order to elucidate the mechanism of regulation, a luciferase reporter assay was performed. The 3'UTR of the ACO1 was cloned into the PsiCheck2 dual luciferase vector. The same UTR with a mutated miR-23a binding site was also cloned. The plasmids, replicated and purified from DH5 α cells, were then transfected with miR-23a (or a control RNA) into HepG2 cells. After 48 hours the proteins were harvested and bioluminescence from the luciferase gene was measured with a luminometer using a dual luciferase assay kit. A summary of this procedure can be seen in Figure 6.

The results from the luciferase reporter assay can be seen in Figure 7. This data showed that overexpression of miR-23a inhibited luciferase activity. Furthermore, the loss of luciferase activity was recovered when the miR-23a binding site was mutated. These results demonstrate that miR-23a is capable of directly targeting ACO1 through its 3' UTR.

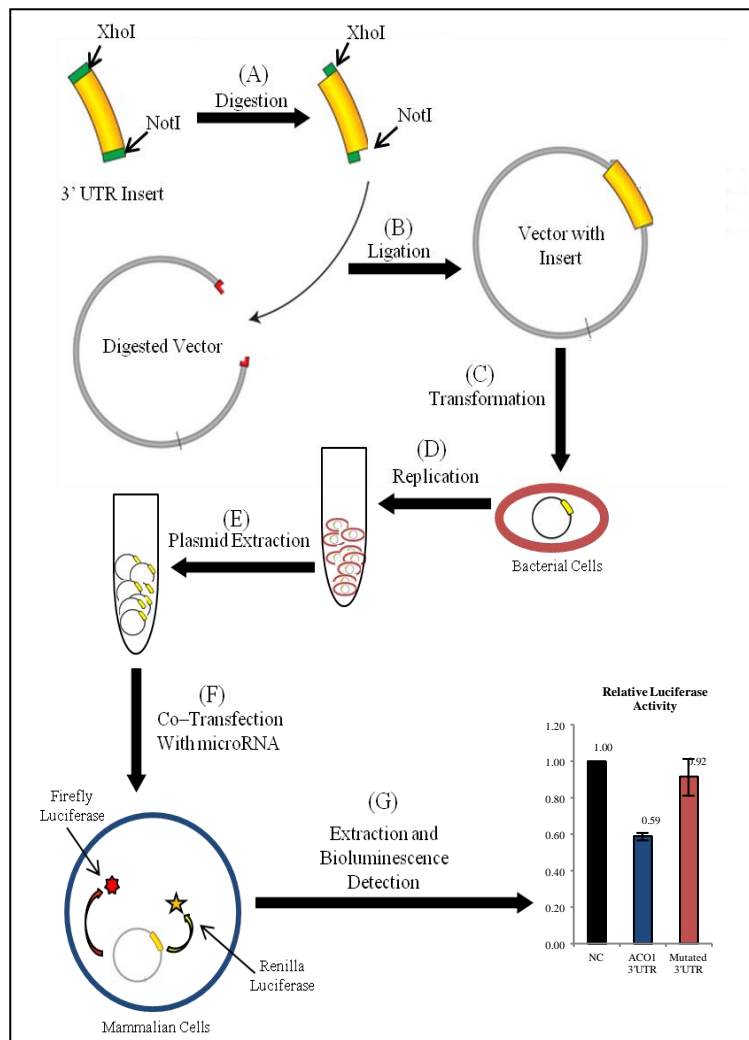


Figure 6 (left): The 3' UTR cloning and luciferase reporter assay procedure. (A) Digestion of insert and vector. (B) ligation of 3' UTR insert into the vector. (C) Transformation of the ligated plasmid into competent cells. (D) Harvest bacterial colonies and allow cells to replicate in isolation. (E) Extract replicated plasmid from bacterial cells. (F) Co-transfect isolated plasmid into mammalian cells with targeting miR. (G) Harvest cellular extracts and detect bioluminescence using dual luciferase assay kit. Some images were modified from addgene.org – PCR Cloning

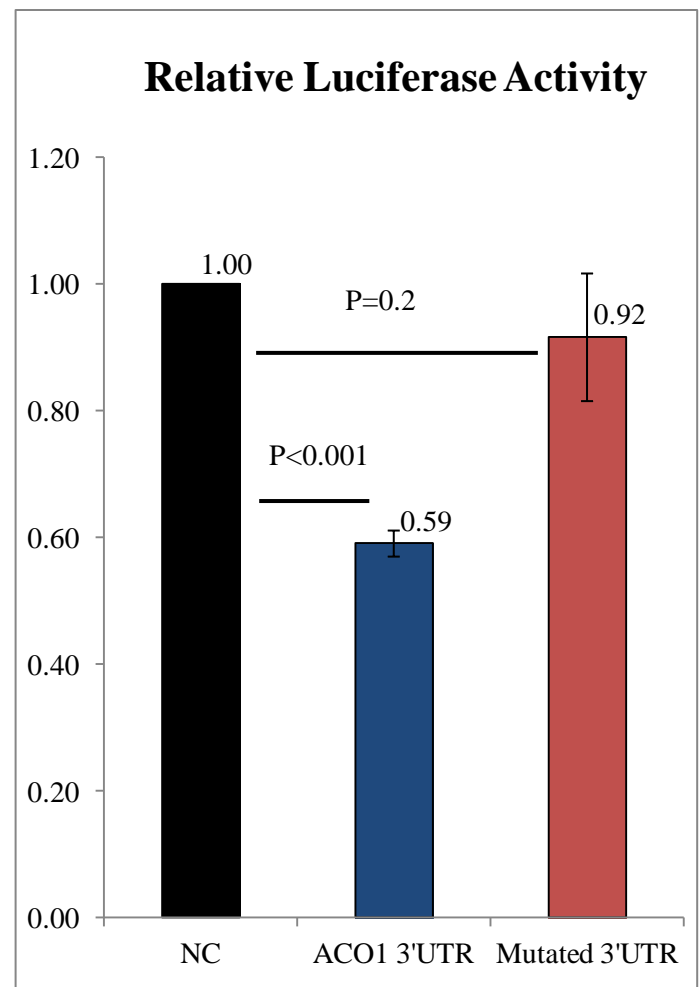


Figure 7 (right): 3'UTR luciferase reporter assay results. HepG2 cells were cotransfected with a dual luciferase expression plasmid and miR-23a. Each plasmid was also cotransfected with a negative control RNA. The relative luciferase readings were normalized to that of the corresponding control. Only one control bar with no error bar is show for simplicity.

Aconitase Activity Assay - Although we know that miR-23a can directly inhibit ACO1 expression, the functional significance of this regulation is unknown. An ACO1 enzymatic activity assay was developed in order to understand this functional significance. This assay measures the rate of the conversion of NADP^+ to NADPH. In this assay citrate is first isomerized to isocitrate via ACO1. An excess of a bacterial isoenzyme for isocitrate dehydrogenase immediately converts the isocitrate to α -ketoglutarate in a reaction reducing NADP^+ to NADPH. This reaction is observed over time in a dynamic spectrometer measuring absorbance at 340 nm; NADPH absorbs light at 340 nm while NADP^+ does not. An illustration of the principles behind this assay can be found below in Figure 8. A detailed protocol for this assay was developed based on existing literature. This protocol can be found in Appendix B.

HepG2 and Hep3B cells were transfected for 48 hours with miR-23a or a control RNA. Protein extracts were then isolated from the cells and utilized in the ACO1 activity assay. The aconitase activity for the miR-23a transfected cells were normalized to that of the corresponding control. It can be seen in Figure 9 that miR-23a overexpression significantly reduced ACO1 activity. This results further demonstrates miR-23a's capability inhibit citrate metabolism.

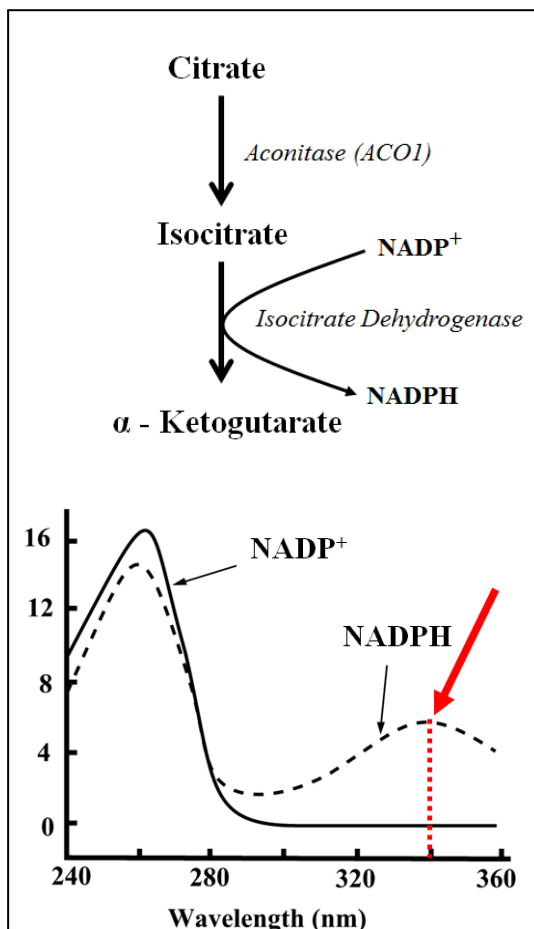


Figure 8 (left): Principle behind the ACO1 activity assay. Citrate is isomerized to isocitrate through aconitase. Isocitrate is then metabolized to α -ketoglutarate through isocitrate dehydrogenase, which reduces NADP^+ to NADPH during the reaction. NADPH is capable of absorbing 340 nm light while NADP^+ is not.

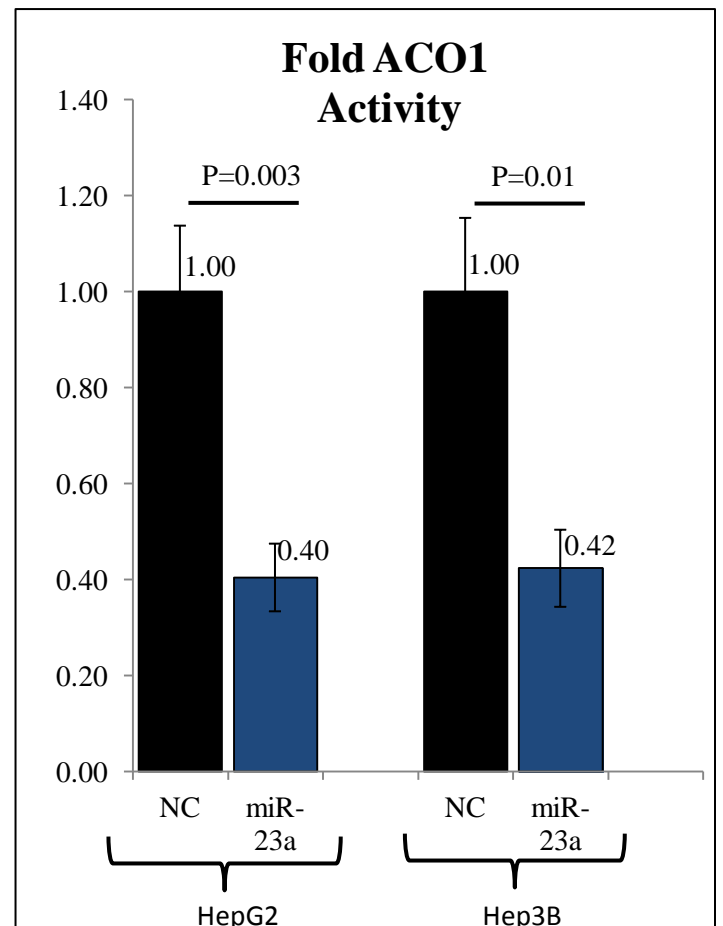


Figure 9 (right): ACO1 activity assay results. HepG2 and Hep3B cells were transfected with miR-23a or a control RNA. Protein extracts were collected 48 hours post transfection and subject to an aconitase activity assay.

Metabolomics

To further analyze the role miR-23a plays in HCC metabolism, metabolic pathways of HCC cell lines will be profiled using stable isotope resolved metabolomic (SIRM) tracing via nuclear magnetic resonance (NMR) and gas chromatography-mass spectroscopy (GC-MS). NMR and GC-MS have been used to trace the metabolism of SIRM in numerous studies.^{16, 17} Stable isotope labeled metabolites (SILM) are structurally similar to their analogous metabolite but are labeled with uncommon isotopes.¹⁸ For example, the SILM $^{13}\text{C}_6$ -Glucose is structurally identical to a typical glucose molecule, except all six of the ^{12}C atoms have been replaced with a ^{13}C isotope. SILMs can be taken up by cells and metabolized in the same fashion as normal metabolites.¹⁸ However, the metabolic products formed from the provided SILM will still carry the isotopic labeled atoms.¹⁸ The compounds that contain these irregular isotopes can then be identified and quantified using NMR and GC-MS.

As of now, these experiments are not yet complete. Over the past several months, a metabolite extracting protocol has been adjusted and optimized for use with the HepG2 cell lines. The optimized protocol is an adaptation of a protocol provided by Teresa Fan, Ph.D. from the University of Louisville. The optimized protocol can be found in Appendix C. Future experiments will utilize this protocol to extract metabolites from HepG2 cells transfected with miR-23a or a control RNA incubated with $^{13}\text{C}_6$ -glucose for 48 hours. The polar and non-polar metabolites will then be analyzed using NMR.

Discussion

Prior research has shown that miR-23a is upregulated in primary HCC and is capable of suppressing hepatic gluconeogenesis through numerous mechanisms.² The goals of this project were to determine if miR-23a is capable of controlling other metabolic pathways in HCC and to elucidate the mechanisms of regulation. A Phenotype MicroArrayTM was initially used to determine which metabolic pathways were strongly affected by miR-23a expression. HepG2 cells transiently transfected with miR-23a demonstrated a significant inhibition of citrate metabolism in the Phenotype MicroArrayTM (Figure 3). This analysis led to a thorough analysis of miR-23a's role in the inhibition of a key enzyme in the citric acid cycle, ACO1.

The enzyme ACO1 is responsible for the first step in citrate metabolism, the isomerization of citrate to isocitrate (Figure 4). The microRNA target prediction algorithm used by TargetScan.org predicted that miR-23a has a binding site on the 3' UTR of ACO1. Based on this prediction, numerous experiments were performed to determine if this regulation occurs. First, RT-PCR was utilized to show miR-23a inhibits ACO1 expression at the mRNA level (Figure 5). Then, the 3' UTR of the ACO1 gene was cloned and utilized in a luciferase report assay. This assay demonstrated that miR-23a is capable of inhibit ACO1 expression by binding directly to the ACO1 3' UTR. Lastly, an aconitase activity assay protocol was developed and utilized to determine the effects of miR-23a on ACO1 activity. This assay confirmed that miR-23a inhibits ACO1 activity in the Hep3B and HepG2 cell lines (Figure 9).

One specific aim of this project was to use NMR based stable isotope resolved metabolomics to confirm miR-23a's ability to alter energy metabolism. However, these experiments are currently in progress. The amount of time required to optimize the metabolite extraction protocol was longer than initially expected. Future experiments will use this protocol to collect metabolites from cells transiently transfected with miR-23a. The metabolites of these cells will then be compared to cells transfected with a control RNA. We expected to see an inhibition of citrate metabolism in the miR-23a transfected cells in addition to some other novel metabolic changes not observed in the Phenotype Microarray.

As tumor cells proliferate, it becomes increasing difficult for these cells to obtain oxygen and nutrients from the blood. This creates a hypoxic environment in which tumor cells must undergo a significant metabolic shift away from oxidative phosphorylation and towards glycolysis for ATP generation. We believe that miR-23a is at least in part capable of facilitating this metabolic shift. By inhibiting hepatic gluconeogenesis and

oxidative phosphorylation, miR-23a helps tumors utilize more intracellular glucose for glycolysis and nucleotide synthesis. This metabolic regulation can be seen in Figure 10. Future experimentation will aim to further understand the role miR-23a plays in HCC metabolism.

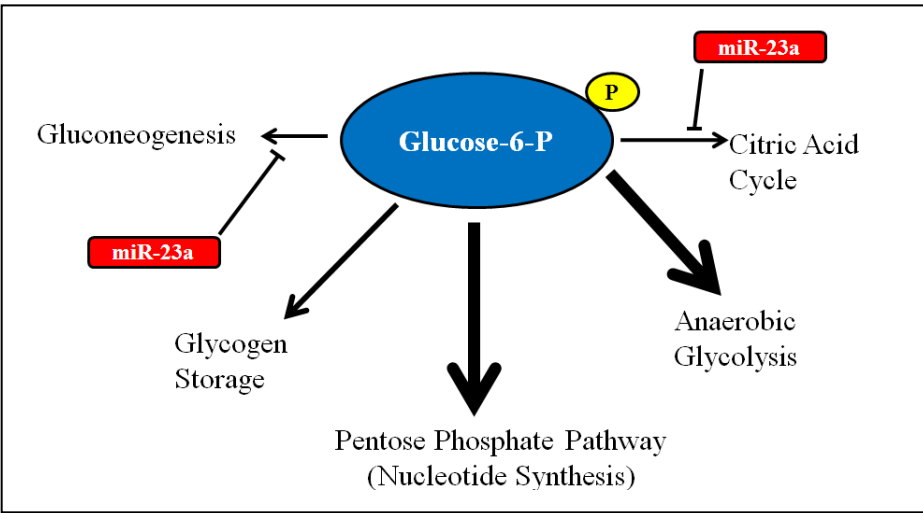


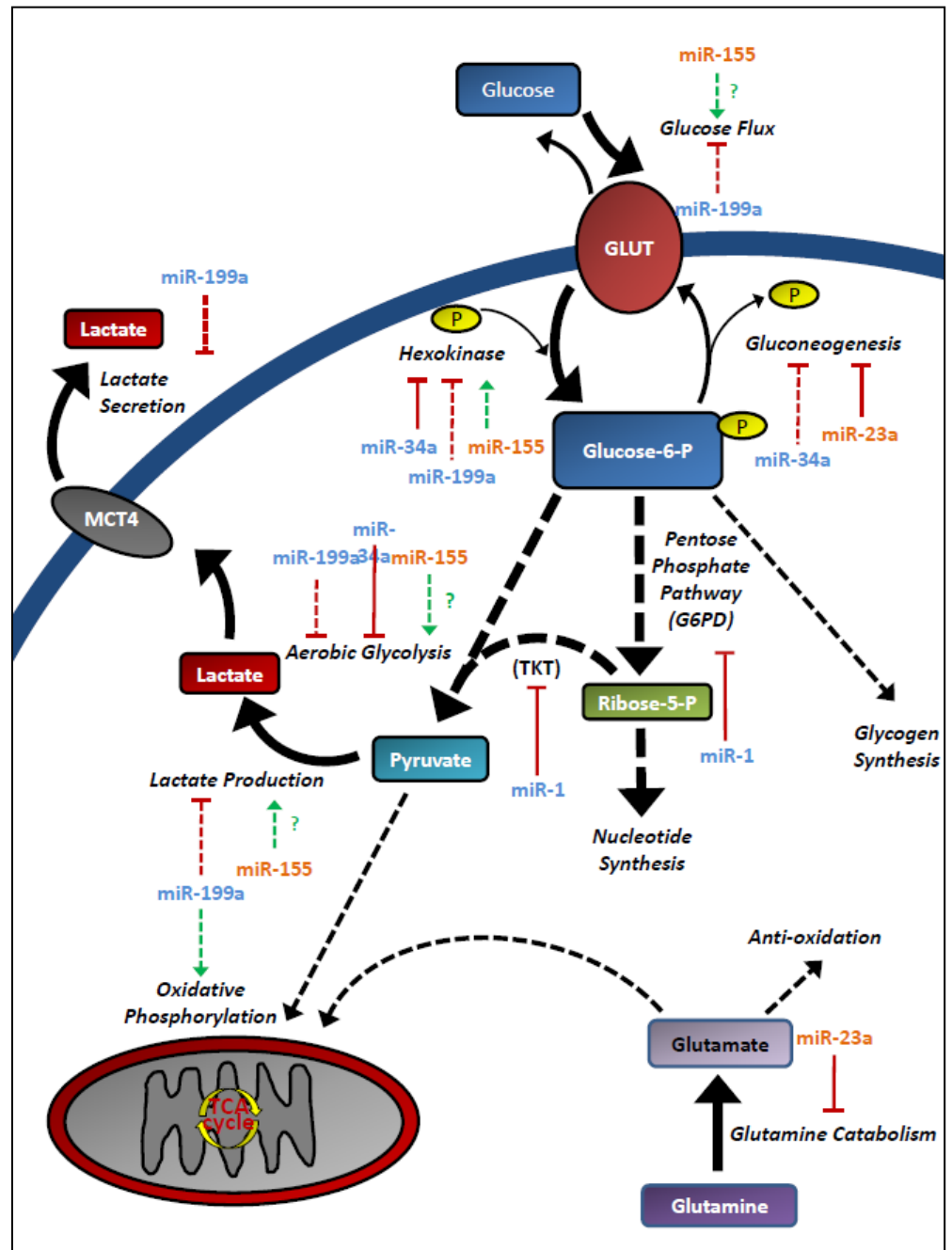
Figure 10: miR-23a mediated shift in HCC glucose metabolism.

Publication

“Regulation of Glucose Metabolism in Hepatocarcinogenesis by MicroRNAs.” *Gene Expression*(2014) In Press

An invited review of the role microRNAs play in HCC carbohydrate metabolism was written for the journal *Gene Expression*. This review, involving an extensive literature search and analysis focused on five microRNAs that have key regulatory functions in HCC: miR-23a, miR-1, miR-34a, miR-155 and miR-199a. The review will be published in April of 2014. Figure 11 shows the primary figure from the review; this figure illustrates the microRNA mediated regulations discussed in the review.

Figure 11: MicroRNA mediated metabolism. The figure shows aspects of microRNA mediated glucose and glutamine metabolism in cancer. Black arrows show the catabolic and anabolic processing of metabolites. The larger black arrows represent pathways that are upregulated in HCC and the broken arrows signify that metabolic intermediates are not shown. MicroRNAs in blue are downregulated in HCC and those in orange are upregulated. MicroRNA mediated regulations are shown in red for inhibition and green for activation. The broken arrows represent mechanisms in which the microRNA does not directly target an enzyme within the specified metabolic pathway. The question marks next to some of the miR-155 regulations indicate mechanisms that are yet to be elucidated. GLUT: glucose transporter, MCT4: monocarboxylate transporter 4, G6PD: glucose-6-phosphate dehydrogenase, TKT: transketolase.



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APPENDIX A

Appendix A - Phenotype MicroArray Dye Optimization

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Last Updated: 6/6/13 RR

This protocol is used to optimize the number of cell and incubation period for using the Biolog Phenotype MicroArray. Each cell line will need to be optimized prior to use. For these experiments, we will only be using Biolog Redox Dye Mix MA. Biolog has shown that this dye is more effective for use with liver cell lines than Dye Mix MB.

Step 1 : Initial Seeding of Cells in Serial Dilution

- Dispense 100 uL of MC-0 media to columns 1 to 6 for rows A and E
 - Recipe for MC-0 media
 - 100 mL Biolog IF-M1 (+aa)
 - 1.1 mL 100x Pen/Strep
 - 0.16 mL 200 mM Glutamine (0.3 mM conc)
 - 5.3 mL Dialyzed FBS (5%)
- Dispense 100 uL of Complete media to columns 1 to 5 for rows A and E
 - Recipe for Complete media (Standard PR-Free DMEM)
 - 43.5 mL Phenol Red-Free DMEM
 - 500 µL 200 mM Glutamine
 - 10 mL FBS (10%)
 - 500 µL 100x Pen/Strep
 - 500 µL 100x Non-Essential Amino Acids
- Prepare cell suspensions
 - Strain cells through a 70 µm cell strainer
 - Divide original cell suspension into two aliquots
 - Wash MC-0 cells in PBS
 - Suspend cells in a small volume of MC-0 and Complete media
 - Obtain cell concentration via hemocytometer
 - Prepare cell suspensions of 4.0×10^5 cells/mL
 - One suspension in MC-0 media
 - One suspension in Complete media
- Dispense 200 µL of MC-0 cell suspension into column 6 for row A
- Starting from column 6, sequentially transfer 100 µL of the cell suspension to adjacent wells to make a twofold serial dilution
 - Mix thoroughly before each transfer
- Withdraw and discard 100 µL of cell solution from rows of column 2

- All wells should now have 100 μ L of solution
- Dispense and dilute Complete cell suspension for row E in the same manner
- Incubate plate for 8 hours at 37° C, 5% CO₂ prior to dye addition

Step 2: Additional Seeding of Cells in Serial Dilution

- Repeat Step 1 three more times for the incubation periods of 6, 4 and 1 hours
 - Allow enough time to prepare cell suspension to ensure incubation times are accurate
 - The 6 hour incubation cell should be seeded into the plate 2 hours after the 8 hour incubation cells, etc.

Step 3: Dye Addition and Detection

- After incubation, add 20 μ L of Biolog Redox Dye Mix MA to all wells.
- Seal the plate with parafilm to reduce loss of CO₂ from the media
- Measure dye reduction with A₅₉₀ and A₇₅₀ measurements using a kinetic micro-plate reader.
 - Set temperature on plate reader to 37° C
 - Use kinetic setting to read every 5 minutes
- Set the plate reader to measure the Vmax of A₅₉₀ minus A₇₅₀
 - Under “Reduced” option
 - Set the maximum points of Vmax to ~4
 - Can adjust if necessary
 - Be sure the “Absolute Values” option is checked on

This protocol is modified from the Biolog handout, *Optimizing the Use of BiologDye Mixes with Mammalian Cell*.

Goals of this optimization

1. Optimal incubation time

After seeding cells onto PM-M1-4 plates, plates must be left to incubate for an extended period of time prior to dye addition (~4-6 hours with dialyzed FBS). This time should be long enough for the cells to metabolize all remaining intracellular energy sources, but not long enough to metabolize all the provided energy within the well.

If the incubation period is too short, cells may still contain intracellular energy stores that were not obtained from the substrate within the well. The metabolism of these energy stores will cause formazan formation. This is known as “background” dye reduction.

If the incubation period is too long, cells may have metabolized all of the provided energy within some wells prior to dye addition. This can lead to reduced dye reduction within these wells.

An optimal incubation period will show a high signal to background ratio. The signal is given by the rate of dye reduction in the well with complete media (high nutrition) after the allotted incubation time. The background is determined by the rate of dye reduction in the well with MC-0 media (no nutrition). This ratio will vary with incubation time and number of cells.

2. Range of linear dye reduction

After seeding into PM-M1-4, cells metabolize the energy source provided within each well. The cells will be able to metabolize some energy sources very quickly, some at a much slower rate, and some may not be metabolized at all. This will be shown by the rate of dye reduction within each well. The linear range of dye reduction will be shorter in wells that exhibit high metabolism than wells that exhibit low metabolism. This means that dye reduction in one well may be out of the linear range prior to detection of reduction in another well.

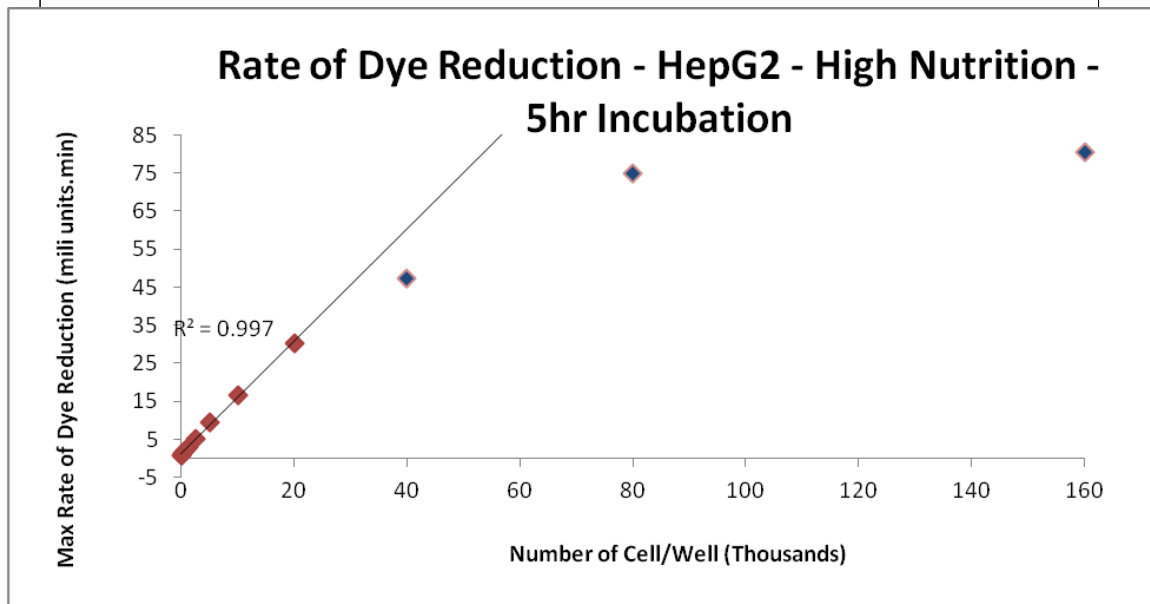
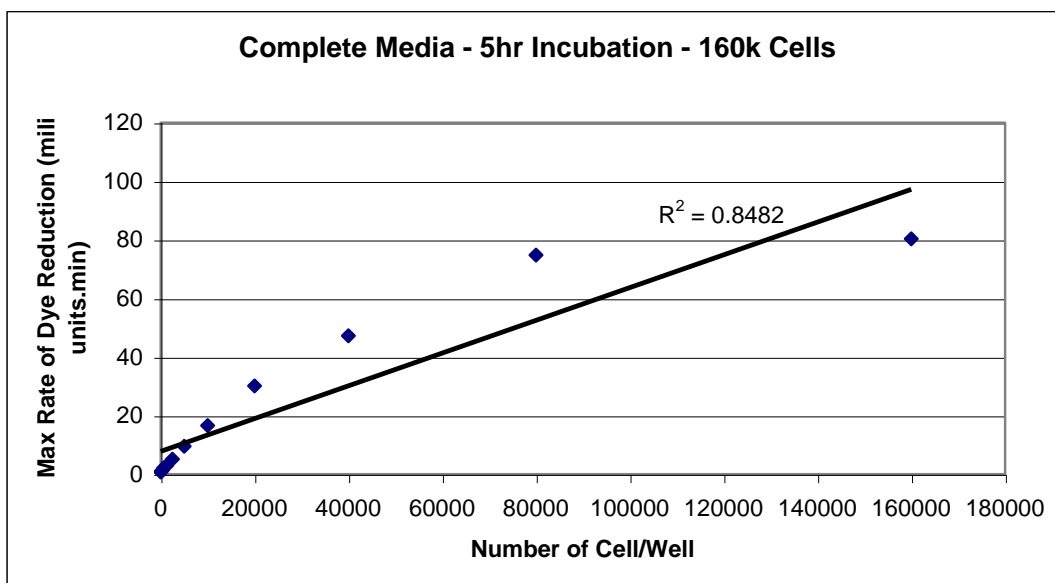
The PM-M1-4 plates will be imaged at multiple time intervals in order to observe linear dye reduction in all wells. In this optimization protocol, 1 hour incubation with Complete media represents a well that provides a high level of nutrition and 1 hour incubation with MC-0 media represent a well that provides a low level of nutrition. The intervals at which the PM-M plates are imaged will be determined based on the rate of dye reduction within these wells.

Dye reduction will be reported in units of $(A_{590}-A_{750})/\text{time}$. This value must be calculated while reduction is in the linear range.

3. Determine Optimal Number of Cells

In order to determine the optimal number of cells, the dye reduction in wells with complete media and long incubation time must be analyzed. A plot of max rate of dye reduction vs. number of cells should be fit to a linear curve. The optimal number of cells will be the greatest number of cells that is within the linear range of reduction. The optimal number of cells will also have a high signal to background ratio.

Below two plots can be seen of the same data. The first contains cell numbers increasing to 160,000 cells/well. The second plot eliminates the wells with high numbers of cells and has a max of 20,000 cells/well. It can be seen that 20,000 cells/well is still within the linear range of dye reduction; however, 160,000 cells/well is not.



		Number Of Cells Per Well						<u>Incubation Time</u>
<u>Media</u>		1.2K	2.5K	5K	10K	20K	40K	
		1	2	3	4	5	6	
MC-0 w/ dFBS	A							8 hours
	B							6 hours
	C							4 hours
	D							1 hour
Complete	E							8 hours
	F							6 hours
	G							4 hours
	H							1 hour

APPENDIX B

Appendix B - RR Aconitase Activity Assay Protocol

Author: Ryan Reyes

Last Updated: 10/25/2013 RR

- Wash Cells
 - Was twice with 5mL ice cold PBS
- Homogenization
 - Scrpae cells in in ~200 uL lysis buffer (see below) – ice cold
 - 50 mM Tris – pH 7.4
 - 0.3 M Sucrose
 - 0.5 mM β -mercaptoethanol
 - 0.5 mM $MnCl_2$
 - 1 mM Citrate
 - Homogenize on ice
 - Pass through a small gauge needle (26G) ten times
 - Can check for cell lysis on a hemocytometer under the microscope
 - Centrifuge 10 min at 3500xG at 4° C to pellet cellular debris
 - Transfer the supernatant to a new tube
- Cytosolic/Mitochondrial Separation
 - Centrifuge at 15,000xG – 15 minutes at 4° C
 - Supernatant is cytosolic fraction
 - Transfer to 1.5 mL tube – keep on ice
 - Pellet contains mitochondrial fraction
 - Resuspend pellet in 200 uL of buffer (see below) – to wash out remaining cytosol
 - 50 mM Tris-HCL – pH 7.6
 - 0.175 M KCl
 - Centrifuge again – same conditions
 - 15,000xG – 15 minutes at 4° C
 - Aspirate supernatant - leave pellet
 - Solubilize mitochondrial protein in 50 uL ice cold buffer (see below)
 - 50 mM Tris-HCl – pH 7.6
 - 1 % Triton X-100
 - 20% Glycerol
 - 0.5 mM β -mercaptoethanol
 - 0.5 mM $MnCl_2$
 - 1 mM Sodium Citrate
 - Keep both fractions on ice or flash freeze in dry ice/ethanol bath and store at -80°C

- Protein Assay
 - Follow BCA protein assay method with BSA standards
 - Dilute protein samples to 10 ug/uL with appropriate lysis buffer
- Aconitase Reactivation
 - Add 40-400 µg of Protein extract to a new 1.5 mL tube
 - 4 x amount of protein to be used in the activity assay
 - Fill to 88 uL with appropriate lysis buffer
 - Add the following agents to each tube in the following order
 - Add 10 uL of 0.5 M DTT
 - Add 1uL of 20 mM Ferrous ammonium sulfate
 - Incubate at 37 degrees for 30 minutes under Nitrogen gas (or other inert gas)

NOTE* Hausladen and Fridovich say perform the assay immediately after addition of reactivating reagents – readings at room temp

- Aconitase Activity Assay
 - Add 25 uL of activated protein extracts to the prepared 96-well plate in triplicate
 - Add 75 uL of Reaction buffer to each well (concentrations are 4/3 that of the final conc)
 - 50 mM Tris-HCl pH 7.4 (do not need to adjust concentration)
 - 40 mM Sodium Citrate (30 mM final concentration)
 - 0.50 mM MnCl₂ (0.5 mM final concentration)
 - 0.267 mM NADP⁺ (0.2 mM final concentration)
 - 2.67 units/mL Isocitric dehydrogenase – (final concentration = 2 units/mL)
 - Record the change in absorbance at 340 nm for 60 minutes (record every 3 minutes)
 - Read at ~25° C
 - Calculate slope of standard curve
 - X axis = absorbance
 - Y axis = nmol isocitrate converted to α-ketoglutarate
 - Ideally: nmol citrate converted to isocitrate = nmol isocitrate converted to α-ketoglutarate
 - Calculate Aconitase activity
 - Calculate units of ACO1 activity/mg protein (assume 1 cm path length)

$$\frac{mU \text{ Activity}}{mg \text{ Protein}} = \frac{\Delta A_{340nm}}{6.22}$$

- Can then normalize to the control sample

APPENDIX C

Appendix C: Metabolite Extraction Protocol

Modified from protocol by Teresa Fan Ph.D.

Last updated: 4/2014 Ryan Reyes

Preparation/Info

- This protocol is optimized for harvesting metabolites from mammalian cells grown on a 10 cm culture dish
- The entire procedure should be done on ice

Procedure

- Wash Cells
 - Remove media and wash cells three times with cold PBS
 - Aspirate PBS after each wash
 - After the last aspiration, tilt plate on a steep angle for ~1 minute. Allow excess PBS to fall into the bottom of the dish and aspirate. Remove as much PBS as possible
- Collect Cells
 - Add 2 mL of cold CH₃CN to cells
 - Store CH₃CN at -20°C prior to extraction
 - Incubate on ice for ~5 minutes
 - Add 1.10 mL of nanopure water + 0.4 mL of 0.2mM Tris-HCL (pH8) to the plate
 - Scrape the cells into the water/CH₃CN solution and transfer into a 15 mL polypropylene tube
 - Add an additional 2 mL of cold CH₃CN and 1.5 mL nanopure water (no Tris) to the plate and scrape/transfer to the same 15 mL tube
 - CH₃CN:water ratio (volume) = 2:1.5
- Fractionation
 - Add 2 mL of CHCl₃ to the 15 mL tube
 - Do not add to the culture plate – may dissolve some of the plastic
 - Add three 3mm glass beads into the tube
 - Wash and autoclave beads prior to use
 - Shake and vortex the tube vigorously for ~3 minutes
 - The mixture will look cloudy
 - Centrifuge the tube at 3000 RCF for 20 minutes at 4°C
 - Transfer most of the top layer (CH₃CN/water - polar) to a new 15 mL centrifuge tube

- Leave some of the top layer to ensure the middle protein layer is not disturb
 - If the middle protein layer is disturbed, recentrifuge
 - Transfer the majority of the lower (CHCl_3 - lipid) layer to a glass screw top vial
 - Leave some of the lower layer
 - Attempt to push away the protein precipitate onto the side of the tube with a pipette tip to access the lower layer
 - Add 10 μL of 100 mM butylated hydroxyl toluene (BHT) to the collected lower lipid fraction as an antioxidant
 - Do not add to the upper polar fraction
 - Transfer the remaining protein precipitate along with the remaining portion of both layers to a 1.5 mL centrifuge tube
 - Cut the tip of the pipette tip to create a wider opening
 - Mix up and down several times before transferring
 - Wash the 15 mL tube and glass beads with 400-1000 μL of CHCl_3 :MeOH:BHT (2:1:1mM)
 - Shake 1.5 mL tube vigorously for ~2 minutes
 - The CHCl_3 :MeOH solution is more effective at extracting lipid metabolites than the initial CH_3CN : CHCl_3 mixture
 - Centrifuge the 1.5 mL tube on a table top centrifuge at max speed (~14,000 RPM) for 5 minutes at 4° C
 - Transfer the majority of the top layer into the same 15 mL tube with the polar fraction
 - Transfer the lower layer into the same glass vial with the lipid fraction
 - May need to use two vials for the same sample
 - Store both fractions at -80° C until lyophilization
- Lyophilization
 - Lyophilize both fractions overnight
 - Reconstitute the samples in deuterated solvent
 - Polar fraction
 - Reconstitute in deuterated water
 - Transfer reconstituted samples into a 1.5 mL tube
 - Lipid Fraction
 - Reconstitute in deuterated Methanol
 - If two glass vials were used, combine the reconstituted samples in a single vial
 - Re-lyophilize overnight
 - Store dried samples at -80°C until NMR analysis
 - Seal with parafilm to prevent the absorption of moisture